REMARKS

This document is submitted in response to the Office Action dated February 21, 2008 ("Office Action"). Claims 27, 32, and 37 are amended to promote clarity. Support for the amendments can be found in original claims 27, 32, and 37. No new matter is added. Claims 1-30, 32-35, 37-40, and 42 are pending. Claims 1-26, 30, 35, 40, and 42 have been withdrawn and claims 27-29, 32-34, and 37-39 are under examination. Reconsideration is requested in view of the remarks below.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner rejected claims 27, 32, and 37 for indefiniteness. See the Office Action, page 2, last paragraph. In view of the above amendments to the three claims, Applicants submit that the claims are definite and that the rejection therefore should be withdrawn.

Rejection under 35 U.S.C. § 103(a)

Claims 27-29, 32-34, and 37-39 were rejected for obviousness on two grounds. Applicants will address each below.

I

The Examiner rejected claims 27-29 and 32-34 over US Patent Application Publication 2003/0148985 by Morrissey *et al.* ("Morrissey"), Paul *et al.*, Natural Biotechnology, May, 2002, 29:505-08 ("Paul"), US Patent No. 6709812 to Stuyver *et al.* ("Stuyver"), and US Patent Application Publication 2004/0029111 to Linnen *et al.* ("Linnen"). See the Office Action, page 3, last paragraph. Applicants respectfully traverse.

The claims cover methods of inhibiting the replication of a virus in a cell or treating an infection with a virus, using an RNA or a DNA vector that contains a nucleic acid encoding the RNA. The methods include introducing into the cell an effective amount of an RNA or of a DNA vector containing a nucleic acid encoding the RNA. The RNA comprises a first nucleotide sequence that hybridizes under stringent conditions to a segment of the gene, and a second nucleotide sequence that is complementary to the first

nucleotide sequence and hybridizes to the first nucleotide sequence to form a duplex structure. The segment has the sequence of SEQ ID NO:3, which is an HBV sequence. That is, the methods target the sequence of SEQ ID NO: 3, which is a segment in the gene encoding HBsAg.

According to the Examiner, "Morrissey *et al.* ... indicated that HBV infection in humans is determined by measuring HBsAg... Linnen *et al.* taught that HBsAg is the first serologic marker for HBV... Stuyver taught that HbsAg gene of HBV is shared among all types of HBV." As such the Examiner concluded that "in order to inhibit the HBsAg segment of the HBV gene in an effective manner, the skilled artisan would have searched for an optimal target sequence that is shared by as many subtypes (or variants) of the HBV genome as possible, and therefore would have reasonably considered ... SEQ ID NO: 3" and then "design [an] RNAi molecule as suggested by Paul *et al.*" See the Office Action, page 7, lines 5-21, emphasis added.

In view of the above cited language, it appears that the Examiner raised the rejection basing on Rationale E "obvious to try" in the "Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co.* v. *Teleflex Inc"* ("Guidelines"). As provided in the Guidelines (emphasis added),

> To reject a claim based on this rationale, Office personnel must resolve the *Graham* factual inquiries. Office personnel must then articulate the following:
>
> (1) a finding that at the time of the invention, there had been a recognized problem or need in the art, which may include a design need or market pressure to solve a problem;
>
> (2) a finding that there had been a finite number of identified, predictable potential solutions to the recognized need or problem;
>
> (3) a finding that one of ordinary skill in the art could have pursued the known potential solutions with a reasonable expectation of success; and
>
> (4) whatever additional findings based on the *Graham* factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness

In this connection, Applicants would like to point out that the Examiner at least has not articulated that there had been "predictable potential solutions to" or "a reasonable expectation of success" using an RNAi molecule that targets SEQ ID NO: 3 to inhibit the replication of HBV.

Indeed, it was well known that RNAi was highly unpredictable. Different RNAi targeting sequences on the HBsAg gene exhibited different efficacies of inhibition of the viral DNA replication and gene expression. See, e.g., page 828, column 2, last paragraph, Cheng *et al.* Biochemical and Biophysical Research Communications 336 (2005) 820–830 (copy attached as "Exhibit A"). In fact, as shown in the specification, out of ten target sequences, two (i.e., HBsAg-1 and HBsAg-2) "did not reduce the serum HBsAg level significantly. And only one of the ten, i.e., SEQ ID NO: 3 (HBsAg-3) inhibited serum HBsAg level completely.

This unpredictability is also evidenced by the follow-up work of the McCaffrey's group, a paper of which was cited by the Examiner in the Office Action. See McCaffrey *et al.* Nat Biotechnol. 2003 Jun;21(6):639-44. Epub 2003 May 12 (copy attached as Exhibit B). Specifically, the McCaffrey's group tested seven RNAi target sequences HBVU6no. 1-7 "on the basis of their conservation among the major HBV genotypes." See, page 639, column 2, lines 5-6, emphasis added. Among the seven, HBVU6no.1 did not inhibit HBsAg. See, page 640, column 1, lines 5-6Also, HBVU6no.4, which was specifically designed to target HBV S-Antigen (see line 12), did not lead to substantial inhibition either. See Figure 2. Thus, it is clear that the mere fact that a sequence is conserved among different HBV genotypes does not provide one of ordinary skill in the art with a reasonable expectation of success. That is, a target sequence that is shared by many subtypes does not inhibit HBsAg in a predictable way.

In view of the above remarks, Applicants submit that the Examiner has not established a *prima facie* case of obviousness. Even a *prima facie* case were established (which Applicants do not agree), it can be rebutted by a showing of an unexpected result. As shown in the specification, an RNA having a sequence corresponding to SEQ ID NO: 3 or a DNA vector that contains a nucleic acid encoding the RNA inhibited serum

HBsAg level completely. See, e.g., page 8, Table 1 and lines 17-18.[1] This complete inhibition was unexpected, as it could not be predicted by the above cited prior art references, alone or combined. Thus, the above-discussed unexpected effect successfully rebutted the obviousness rejection and the claims at issues are non-obvious.

II

Claims 37-39 were rejected as obvious over Morrissey in view of Stuyver, Linnen, and McCaffery *et al.* Nature, July 2002, 418:38-39 ("McCaffery"). See the Office Action, page 8, lines 6-8. For at least the same reasons set forth above in part I, Applicants submit that these claims are also non-obvious.

III

In the previous response, Applicants presented similar unexpected results. However, the Examiner countered that the results were not persuasive on four grounds. See page 10, line 7 to page 11, line 2. Applicants respectfully traverse.

First, the Examiner alleged that the inhibition achieved by HBsAg-3 "is not at all 'unexpected.'" To support this allegation, the Examiner referred to inhibition data of four other sequences that were obtained by the Applicants and presented in the specification. See the Office Action, page 10, lines 7-10. In other words, the Examiner was comparing the results of the invention with the results of the invention.

Applicants respectfully traverse. As the Examiner is aware, MEPE specifically directs examiners not to compare the results of the invention with the results of the invention , but compare the results of an invention with the closest prior art. Specifically, MPEP 716.02(e) III provides (emphasis added),

> evidence of unexpected results must compare the claimed invention with the closest prior art, applicant is not required to compare the claimed invention with subject matter that does not exist in the prior art. *In re Geiger*, 815 F.2d 686, 689, 2 USPQ2d 1276, 1279 (Fed. Cir. 1987) (Newman, J., concurring) (Evidence rebutted *prima facie* case by comparing claimed invention with the most relevant prior art. Note that the majority held the Office failed to establish a *prima facie* case of obviousness.); *In re Chapman*, 357 F.2d 418,

[1] According to MPEP2144.08 IIB, rebuttal evidence and arguments can be presented in the specification, *In re Soni*, 54 F.3d 746, 750, 34 USPQ2d 1684, 1687 (Fed. Cir. 1995), by counsel, *In re Chu*, 66 F.3d 292, 299, 36 USPQ2d 1089, 1094-95 (Fed. Cir. 1995).

> 148 USPQ 711 (CCPA 1966) (Requiring applicant to compare claimed invention with polymer suggested by the combination of references relied upon in the rejection of the claimed invention under 35 U.S.C. 103 "would be requiring comparison of the results of the invention with the results of the invention." 357 F.2d at 422, 148 USPQ at 714.).

In view of the clear guidance from MPEP, Applicants submit that the Examiner's allegation is unstable. To the extent that the Examiner "[r]equir[s]e applicant to compare ... the results of the invention with the results of the invention," the Examiner failed to establish a *prima facie* case of obviousness in the same manner as "the Office failed to establish a *prima facie* case of obviousness" in the cases cited above.

Second, it seems to be the Examiner position that the data presented in the application is not creditable since "the ELISA method [used in the application] ... is a semi-quantitative method." See the Office Action, page 10, lines 11-13. Applicants note that the prior art also used the ELISA method. See, e.g., Morrissey, paragraph [223]; McCaffrey *et al.* (Exhibit B), p640, column 1, lines 4-6. Thus, the Examiner's position is not tenable.

Third, the Examiner asserted that "there is no statistical analysis." See the Office Action, page 10, lines 13-14. Applicants would like to point out that all the data were statistically analyzed and the standard deviations were included in all of the data tables in the specification. See Tables 1-5. Thus, the Examiner assertion is baseless.

Finally, the Examiner asserted that, because SEQ ID NO: 3 was "well-conserved by 35 different HBV genome sequence," "a strong level of inhibition on HBsAg expression would have been reasonably expected by any person of ordinary skill in the art." See, page 10, lines 14-22. Applicants disagree. As discussed above in part I and demonstrated by McCaffrey *et al.* (Exhibit B), the mere fact that a sequence is conserved among different HBV genotypes does not provide one of ordinary skill in the art with a reasonable expectation of success.

Conclusion

It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue or comment does not signify agreement

with or concession of that rejection, issue or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, except as specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

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Respectfully submitted,

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Jianming Hao, Ph.D.
Reg. No. 54,694

Customer No. 69713
Occhiuti Rohlicek & Tsao LLP
10 Fawcett Street
Cambridge, MA 02138
Telephone: (617) 500-2512
Facsimile: (617) 500-2499
34440.doc

Exhibit A

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Therapeutic inhibition of hepatitis B virus surface antigen expression by RNA interference

Tsung-Lin Cheng [a,1], Wen-Wei Chang [a,1], Ih-Jen Su [b], Ming-Derg Lai [a,c], Wenya Huang [a,d], Huan-Yao Lei [a,e,*], Wen-Tsan Chang [a,c,*]

[a] *Institute of Basic Medical Sciences, National Cheng Kung University Medical College, 1, University Road, Tainan 701, Taiwan, ROC*
[b] *Division of Clinical Research, National Health Research Institutes, 138, Sheng-Li Road, Tainan 701, Taiwan, ROC*
[c] *Department of Biochemistry and Molecular Biology, National Cheng Kung University Medical College, 1, University Road, Tainan 701, Taiwan, ROC*
[d] *Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University Medical College, 1, University Road, Tainan 701, Taiwan, ROC*
[e] *Department of Microbiology and Immunology, National Cheng Kung University Medical College, 1, University Road, Tainan 701, Taiwan, ROC*

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Abstract

RNA interference (RNAi) mediated inhibition of virus-specific genes has emerged as a potential therapeutic strategy against virus induced diseases. Human hepatitis B virus (HBV) surface antigen (HBsAg) has proven to be a significant risk factor in HBV induced liver diseases, and an increasing number of mutations in HBsAg are known to enhance the difficulty in therapeutic interventions. The key challenge for achieving effective gene silencing in particular for the purpose of the therapeutics is primarily based on the effectiveness and specificity of the RNAi targeting sequence. To explore the therapeutic potential of RNAi on HBV induced diseases in particular resulted from aberrant or persistent expression of HBsAg, we have especially screened and identified the most potent and specific RNAi targeting sequence that directly mediated inhibition of the HBsAg expression. Using an effective DNA vector-based shRNA expression system, we have screened 10 RNAi targeting sequences (HBsAg-1 to 10) that were chosen from HBsAg coding region, in particular the major S region, and have identified four targeting sequences that could mediate sequence specific inhibition of the HBsAg expression. Among these four shRNAs, an extremely potent and highly sequence specific HBsAg-3 shRNA was found to inhibit HBsAg expression in mouse HBV model. The inhibition was not only preventive in cotransfection experiments, but also had therapeutic effect as assessed by post-treatment protocols. Moreover, this HBsAg-3 shRNA also exhibited a great potency of inhibition in transgenic mice that constitutively expressed HBsAg. These results indicate that HBsAg-3 shRNA can be considered as a powerful therapeutic agent on HBsAg induced diseases.

Keywords: Hepatitis B virus; HBV surface antigen; RNA interference; Short hairpin RNA; Hydrodynamics-based transfection; HBsAg transgenic mouse

Small interfering RNAs (siRNAs) have evolved as powerful gene silencing reagents that efficiently mediate sequence-specific degradation of homologous RNA transcripts through a process known as RNA interference (RNAi) [1–3]. siRNAs are 21- to 23-nucleotide (nt) short RNA duplexes with 2 nt 3′ overhangs [4–6], which can be introduced by exogenous delivery of synthetic siRNAs [7,8] or produced by endogenous DNA vector-based expression systems [9–12]. RNAi technology has been widely used not only as an extremely powerful strategy for reverse functional genomics [13–15], but also as an effectively potent method for gene silencing-based therapeutics [16,17]. RNAi-mediated inhibition of virus infection or replication has been reported for numerous

* Corresponding authors. Fax: +886 6 2097825 (H.-Y. Lei), +886 6 2741694 (W.-T. Chang).
E-mail addresses: hylei@mail.ncku.edu.tw (H.-Y. Lei), wtchang@mail.ncku.edu.tw (W.-T. Chang).
[1] These two authors contributed equally to this work.

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viruses, including several important human pathogens such as poliovirus [18], HIV-1 [10,19], hepatitis C virus [20,21], influenza virus A [22], dengue virus [23,24], and hepatitis B virus [25,26].

Hepatitis B virus (HBV) is a small enveloped DNA virus that infects almost exclusively the liver cells [27]. An estimated 350 million people worldwide are chronically infected with HBV, and these individuals have a high relative risk of developing liver cirrhosis and eventually hepatocellular carcinoma (HCC) [28–30]. HBV contains a partially double-stranded circular genome with only about 3.2 kb in length that encodes four overlapped open reading frames (ORFs), including polymerase (HBV P), core and secreted e antigens (HBcAg and HBeAg), surface (pre-S and S) antigens (HBsAg), and X protein (HBx) [27]. These proteins play important roles in transcriptional regulation, viral package, reverse transcription, and viral DNA recycling. Accumulating evidences indicate that HBV and its gene products may involve in the multistep progression of HBV-related tumorigenesis [31]. Among the four viral proteins, the HBsAg and HBeAg are two important risk factors for HCC [32].

Liver injury induced by the HBV infection is not due to virus replication or cytopathic effects on the infected cells, but to the immune response against cells expressed viral antigens [33]. In addition, substantial evidence suggests that differences in outcome of HBV infection are determined by variations in the immune responses and expression of the HBV antigens in liver tissue, which reflects the replicative status and the disease activity of chronic HBV infection. Although HBV infection can be effectively prevented by vaccination of the HBsAg, the current treatments for chronic HBV infection are restricted to the use of interferon-α and nucleoside analogues that aim to block the HBV replication [34–38]. However, in the natural course of chronic HBV infection, the early active replication stage would gradually evolve into the late non-replication stage, which usually only associated with the HBsAg expression. Moreover, our previous studies have shown that emergence and accumulation of the preS mutants in serum and liver might account for the life-long persistence of HBV infection and might also confer growth advantages for the clustering proliferation of hepatocytes harboring the preS2 mutant [39–42].

Recently, several groups have reported that HBV replication and expression could be inhibited by directly applying either exogenously synthetic siRNAs or endogenously DNA vector-expressed shRNAs [25,26,43,44]. They demonstrated the efficacy and specificity of the inhibition in either human hepatoma cell lines or mouse HBV model by hydrodynamics-based transfection of viral DNA or HBV transgenic mice. However, the key challenge for achieving effective gene silencing in particular for the purpose of the therapeutics is primarily dependent on the effectiveness and specificity of the RNAi targeting sequence. To explore the therapeutic potential of RNAi technology on HBV induced diseases in particular caused by aberrant or persistent expression of HBsAg, we have especially screened and identified the most potent and specific RNAi targeting sequence located in the major S coding region. The targeting sequence is nineteen nucleotides in length and has not been shown any therapeutic intervention induced mutations. Using an effective DNA vector-based shRNA expression system, pSUPER [9], we have demonstrated that HBsAg-3 shRNA not only inhibited the HBsAg expression in the in vitro human hepatoma cell line, but also suppressed the HBsAg expression in the in vivo mouse HBV model induced by hydrodynamics-based transfection of viral DNA, and in particular in HBsAg transgenic mice. These results clearly indicate that HBsAg-3 shRNA can be considered as an effective and specific therapeutic agent, as well as has a great potential for clinical application in patients with chronic HBV infection.

Materials and methods

Plasmids. p(3A)SAg containing HBsAg gene was kindly provided by Dr. C.-C. Lu (Department of Pathology, National Cheng Kung University, Tainan, Taiwan) [40] and pHBV3.6 containing all HBV ORFs was kindly provided by Dr. L.-P. Ting (Department of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan) [45]. pSUPER containing human RNA polymerase-III H1 RNA gene promoter was kindly provided by Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam, Netherlands) [9]. Plasmid vectors were constructed using standard molecular cloning techniques. The human RNA polymerase-III U6 gene promoter was PCR-amplified using synthetic oligonucleotides, HsU6-S: 5′-GGAATTCAAGGTCGGGCAGGAAGAG G-3′ and HsU6-AS: 5′-CCCAAGCTTCCATCGATGTTTCGTCCTTTCC ACAAGATAT-3′, and cloned into an *Eco*RI/*Cla*I restriction site of a pGEM-7Zf(+) vector (Promega, Madison, WI, USA). Ten RNAi target sites (HBsAg-1 to 10) were chosen for being directed against the HBsAg mRNA. Oligonucleotides were purchased from commercial suppliers. A general strategy for constructing pSUPER-shRNA expression vector involved ligating an annealed oligonucleotide duplex into *Bgl*II/*Hin*dIII restriction sites of the pSUPER vector. The reconstructed HBsAg expression vectors, including p(3A)SAg-luc+, pHBV3.6-luc+, p(3A)SAg-EGFP, and pHBV3.6-EGFP, contained the firefly luciferase gene from pGL3-Promoter (Promega) and the EGFP gene from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA) inserted into the p(3A)SAg and pHBV3.6 vectors, respectively. All plasmids were prepared with Plasmid Maxi Purification Kit (Viogene, Sunnyvale, CA, USA).

Cell line and transfection. The human hepatoma derived cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Rockville, MD, USA) supplemented with heat-inactivated 10% fetal calf serum (Biological Industries, Ashrat, Israel) and 1% antibiotic/antimycotic solution (Gibco-BRL) at 37 °C in a humidified incubator with 5% CO_2. Twenty-four hours before transfection, cells were trypsinized and seeded in six-well culture plates at 1×10^5 cells per well. The cells were transiently cotransfected with 0.5:1.5 or 1:1 μg HBsAg and HBsAg shRNA expression vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The medium was replaced with a fresh medium 5 h after transfection, and cells were harvested 60 h after transfection. Experiments were performed in triplicate.

Mice. Breeder mice of BALB/c strain were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Japan, (Atsugi, Japan). They were fed standard laboratory chow and water ad libitum in the animal facility. The animals were raised and cared for following the guidelines set up by the National Science Council of the Republic of China. Eight- to 12-week-old mice (20–25 g body weight) were used in all experiments.

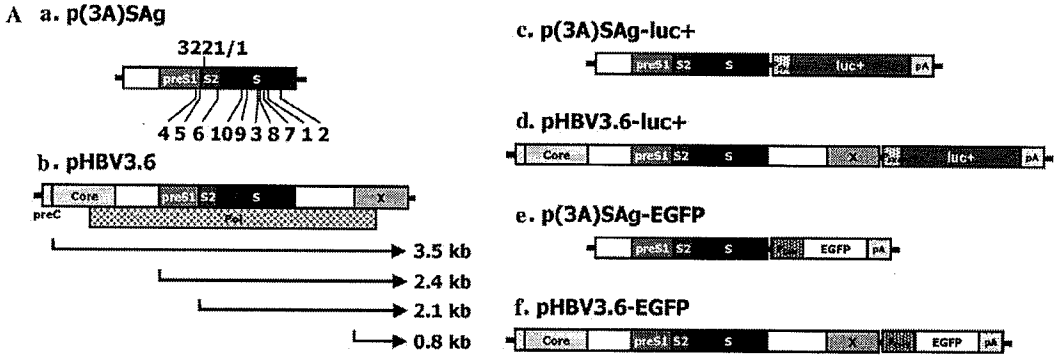
Transgenic mice. The p(3A)SAg [40] plasmid was used to generate transgenic mice in The Animal Center of National Cheng Kung University

[42]. The plasmid was microinjected into embryos of FVB/N mice. All microinjected embryos were implanted into pseudopregnant female mice. Mice carrying the transgene were selected by PCR detection of the pre-S gene. Mice with the HBsAg transgene were next tested for HBsAg protein production in the sera and livers by enzyme-linked immunosorbent assay (ELISA) kit (General Biological, Hsinchu, Taiwan).

Hydrodynamics-based transfection. All the plasmid DNAs were injected into the tail vein of 8- to 12-week-old mice in a volume of Ringer's solution (154 mM NaCl, 5.63 mM KCl, and 2.25 mM $CaCl_2$) equivalent to 11% of the body weight of the mouse (e.g., 2.2 ml for mouse of 20 g). The total volume was delivered within 5–7 s following the hydrodynamics-based transfection protocol described previously [46]. The percentage of hepatocytes that expressed injected DNA was determined by using β-galactosidase gene as a reporter. Histochemical analysis revealed that approximately 20% of hepatocytes express the transgene after hydrodynamics-based transfection of 10 μg pCMVβ expression vector (BD Biosciences Clontech).

Detection of HBsAg and anti-HBcAg antibody in mouse sera. Mouse sera were collected at different days after hydrodynamics-based transfection of p(3A)SAg (for HBsAg detection) or pHBV3.6 (for HBsAg and anti-HBcAg antibody detection) and pSUPER-shRNA expression vectors. The levels of HBsAg expression were determined by ELISA (General Biological). The limitation of this HBsAg assay is 0.25 ng/ml. Anti-HBcAg antibody was detected by competition ELISA (General Biological). The cutoff value of anti-HBcAg antibody assay was calculated by $[0.4 \times (A_{490}$ of negative control$) + 0.6 \times (A_{490}$ of positive control$)$. All the assays used standard protocols and were compared to positive and negative control sera provided by the manufacturer.

Immunofluorescent and immunohistochemical staining of HBsAg. For immunofluorescence staining, cells grown on glass coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100 at 60 h after in vitro plasmid transfection. Immunostaining was performed according to standard protocols. For immunohistochemical staining, liver tissues were embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen, and 4 μm cryosections were made using cryostats (Leica CM 1800, Nussloch, Germany). The sections were fixed by cold acetone and endogenous peroxidase was inhibited by 3% H_2O_2/phosphate buffered saline (PBS). HBsAg was



B a. HBsAg-3 shRNA

5′-GGUAUGUUGCCCGUUUGUC
3′-UUCCAUACAACGGGCAAACAG

b. HBsAg siRNAs

HBsAg-1 (524-542)	**HBsAg-2 (637-655)**
CCUGCACGACUCCUGCUCAUU	UACCUAUGGGAGUGGGCCUUU
UUGGACGUGCUGAGGACGAGU	UUAUGGAUACCCUCACCCGGA
HBsAg-3 (460-478)	**HBsAg-4 (3162-3180)**
GGUAUGUUGCCCGUUUGUCUU	GGCAGCCUACUCCCAUCUCUU
UUCCAUACAACGGGCAAACAG	UUCCGUCGGAUGAGGGUAGAG
HBsAg-5 (3192-3210)	**HBsAg-6 (125-143)**
GAGACAGUCAUCCUCAGGCUU	UCUCCGCGAGGACUGGGGAUU
UUCUCUGUCAGUAGGAGUCCG	UUAGAGGCGCUCCUGACCCCU
HBsAg-7 (500-518)	**HBsAg-8 (476-494)**
CAACCAGUACGGGACCAUGUU	GUCCUCUAAUUCCAGGAUCUU
UUGUUGGUCAUGCCCUGGUAC	UUCAGGAGAUUAAGGUCCUAG
HBsAg-9 (425-443)	**HBsAg-10 (326-344)**
GCCUCAUCUUCUUAUUGGUUU	CCUCCAAUCACUCACCAACUU
UUCGGAGUAGAAGAAUAACCA	UUGGAGGUUAGUGAGUGGUUG
HBsAg-3(mut-9)	**HBsAg-3(mut-2,9)**
GGUAUGUUACCCGUUUGUCUU	GAUAUGUUACCCGUUUGUCUU
UUCCAUACAAUGGGCAAACAG	UUCUAUACAAUGGGCAAACAG

Fig. 1. HBsAg expression vectors and RNAi targeting sites. (A) HBV expression vectors and relative location of RNAi targeting sites. (a) p(3A)SAg was constructed specifically for HBsAg expression, and the 10 targeting sites were selected and marked by numbers within the HBsAg coding region. (b) pHBV3.6 contained the full-length HBV genome that encodes preC (pre-core antigen), Core (HBcAg), preS1 (large pre-surface antigen), S2 (middle pre-surface antigen), S (major surface antigen), X (HBx), and Pol (polymerase). The four HBV transcripts are shown below the HBV open reading frames. (c) p(3A)SAg-luc+ and (d) pHBV3.6-luc+ vectors were constructed including pGL3-promoter expression cassette for reference protein firefly luciferase expression. (e) p(3A)SAg-EGFP and (f) pHBV3.6-EGFP vectors were constructed containing pEGFP-N1 expression cassette for reference protein EGFP expression. (B) Predicted secondary structure of pSUPER-HBsAg-3 transcript (a), and putative siRNAs derived from 10 wild-type and two mutated HBsAg-3 shRNAs, HBsAg-3(mut-9) and HBsAg(mut-2,9) (b). Nucleotides in boxed indicate the mutated sequences in HBsAg-3 siRNA.

detected with sheep polyclonal anti-HBsAg antibody (Serotec, Oxford, UK). Secondary antibody used was biotinylated anti-sheep and then incubated with peroxidase-conjugated streptoavidin (DAKO, Carpinteria, CA, USA). Peroxidase staining of red color was developed by aminoethyl carbazole substrate (Zymed Laboratories, San Francisco, CA, USA) and counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Western blot analysis of HBsAg. Cells or liver tissues were harvested at the indicated time points after in vitro plasmid transfection or in vivo hydrodynamics-based transfection and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 2 mM EDTA, and 1% NP-40) containing protease inhibitors (Roche, Mannheim, Germany). Total protein extracts (30 μg per lane) were resolved on a 10% SDS–polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, USA), and incubated with a commercial available specific antibody and anti-EGFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin monoclonal antibody (Sigma Chemical, Saint Louis, MO, USA), followed by incubation with horseradish peroxidase-conjugated anti-IgG (species dependent) secondary antibody. The bands were visualized by using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Northern blot analysis of HBV transcripts. Thirty micrograms of total RNA purified from mouse liver after hydrodynamics-based transfection of pHBV3.6-EGFP and pSUPER-shRNA expression vectors by using TRI Reagent (Molecular Research Center, City, OH, USA) on nylon membrane was hybridized with ^{32}P-labeled specific DNA probe (prepared from *Bst*EII/*Eco*RV digested p(3A)SAg containing sequence 2823–1045 of HBV adw2 subtype) at 37 °C overnight. The hybridized bands were visualized by autoradiography.

Detection of HBV DNA in mouse liver and serum. The cytoplasmic DNA of mouse liver was purified as described previously by Guidotti et al. [47]. Cytoplasmic DNAs isolated from 30 mg liver tissues were separated on agarose gel electrophoresis, transferred to nylon membrane, and hybridized with, ^{32}P-labeled specific DNA probe (located at nt 181–895 of HBV adw2 subtype) at 56 °C overnight. The hybridized bands were visualized by autoradiograph. Mouse sera were collected at day 2 after hydrodynamics-based transfection of pHBV3.6 and pSUPER-shRNA expression vectors. Before and after treatment with DNase I (20 U, >12 h) to eliminate the plasmid DNA, HBV DNA was purified from 200 μl mouse serum by viral DNA/RNA isolation kit (Maxim Biotech, San Francisco, CA, USA) and 5 μl of isolated DNA solution was used to detection by PCR analysis. The primer pairs (SP2-d1, 5′-GCGGGTCA CCATATTCTTGG-3′, and SP2-d4, 5′-GAGTCTAGACTCTGCGGT AT-3′) were used to amplify the preS2 region of HBsAg gene.

Results

Screening selection of effective RNAi targeting sequences from HBsAg coding region

To directly inhibit the expression of the HBsAg gene, 10 RNAi targeting sequences were chosen from HBsAg coding region, in particular the major S region (Fig. 1A), on the basis that the sequences had not been shown any therapeutic intervention induced mutations. In addition, to efficiently induce RNAi mediated inhibition of the HBsAg expression, an effective DNA vector-based shRNA expression system, pSUPER [9], was used. An example of the predicted secondary structure of pSUPER-HBsAg-3 transcript, HBsAg-3 shRNA, and sequences of the putative siRNAs derived from HBsAg-1 to HBsAg-10 shRNAs including mutated HBsAg-3 shRNAs, HBsAg-3(mut-9), and HBsAg-3(mut-2,9), is shown in Fig. 1B. Moreover, to normalize the transfection variation and effectively evaluate the efficacy of the RNAi targeting sequences against HBsAg expression, we have reconstructed two different HBsAg expression vectors, p(3A)SAg [40] and pHBV3.6 [45], for which they contained additionally either firefly luciferase (p(3A)SAg-luc+ and pHBV3.6-luc+) or EGFP (p(3A)SAg-EGFP and pHBV3.6-EGFP) expression cassette as a reference protein expression system (Fig. 1A).

To assess the inhibition effects of 10 selected HBsAg-1 to HBsAg-10 shRNAs on HBsAg expression, human hepatoma cell line Huh7 cells were cotransfected with 0.5:1.5 or

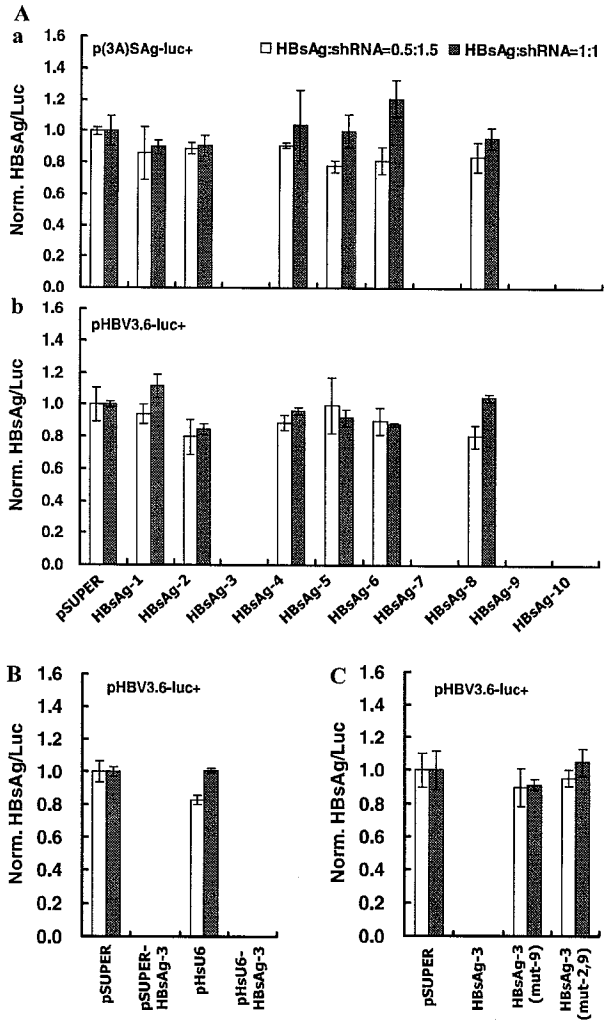


Fig. 2. Screening selection of effective HBsAg shRNAs for suppressing HBsAg expression in human hepatoma cell line. Inhibition effects of 10 selected HBsAg-1 to HBsAg-10 shRNAs (A), HBsAg-3 shRNA derived from pHsU6 expression vector (B), and HBsAg-3(mut-9) and HBsAg-3(mut-2,9) two mutated HBsAg-3 shRNAs (C) on HBsAg expression in Huh7 cells. HBsAg-3 targeting sequence was mutated to give a 1- or 2-base pairs substitution at position 2 or 9. Huh7 cells were cotransfected with either 0.5:1.5 or 1:1 μg HBsAg and HBsAg shRNA expression vectors as indicated by Lipofectamine 2000. At 60 h post-transfection, the expression levels of HBsAg and luciferase activity in the total protein extracts were determined by HBsAg ELISA kit and luciferase assay. The HBsAg/firefly luciferase ratio was normalized and calculated against the control vector (pSUPER or pHsU6). The plotted data were averaged from three independent experiments and the bars represent ± SD.

1:1 μg p(3A)SAg-luc+ and pSUPER-HBsAg expression vectors by Lipofectamine 2000. The expression levels of HBsAg and firefly luciferase activities in total protein extracts were determined at 60 h post-transfection. Among the 10 pSUPER-shRNA expression vectors, the pSUPER-HBsAg-3, 7, 9, and 10 four constructs exhibited a complete inhibition of the p(3A)SAg-luc+-induced HBsAg protein production in both the cotransfection of 0.5:1.5 and 1:1 μg ratios. While the control vector pSUPER or pSUPER-HBsAg-1, 2, 4, 5, 6, and 8 six constructs were ineffective in the two transfected concentrations (Fig. 2A). When the HBsAg expression vector from

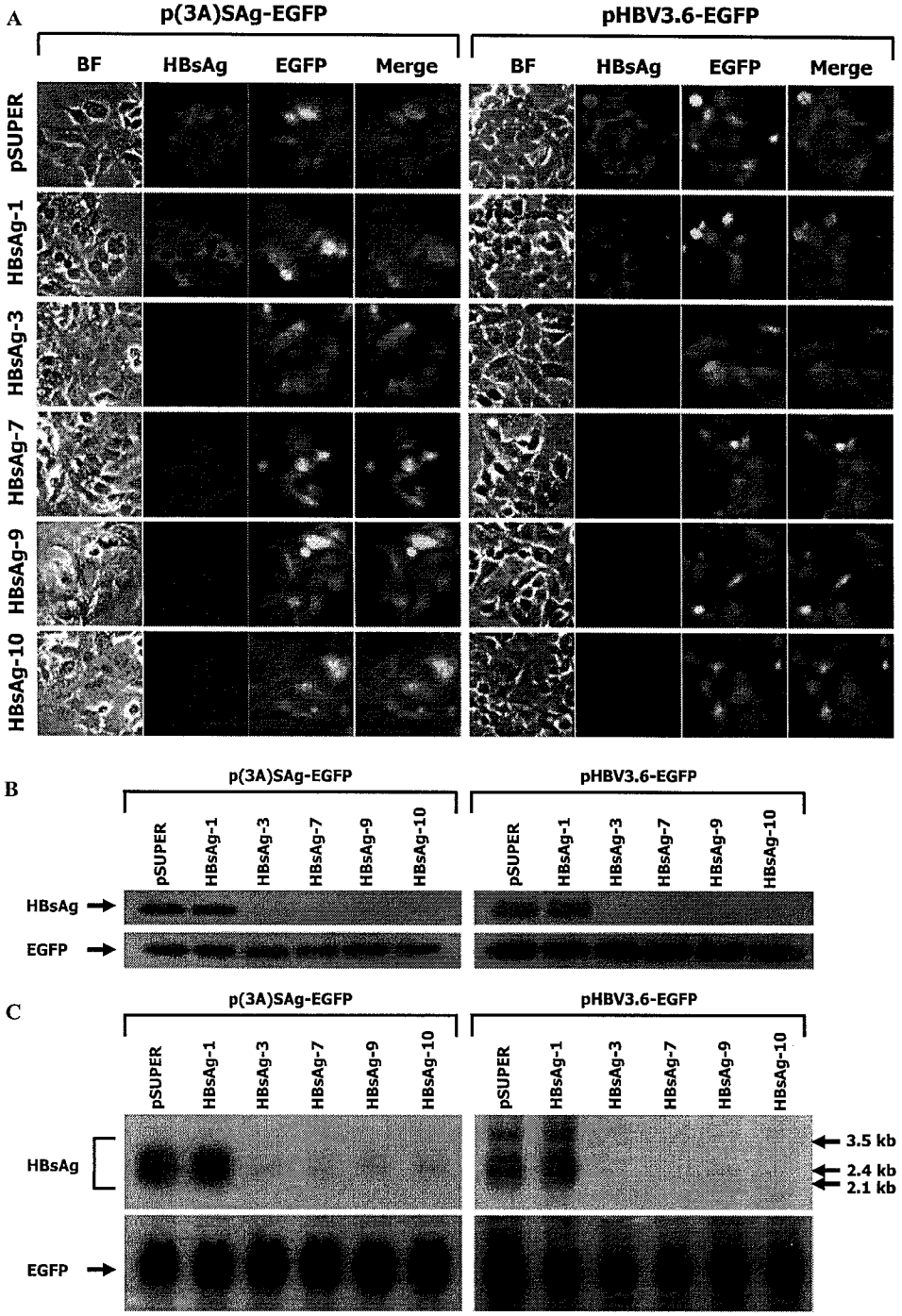


Fig. 3. Immunostaining, Western and Northern blot analyses of HBsAg-3, 7, 9, and 10 shRNAs on in vitro inhibition of HBsAg expression in human hepatoma cell line. Huh7 cells were cotransfected with 0.5:1.5 μg HBsAg and HBsAg shRNA expression vectors as indicated by Lipofectamine 2000. At 60 h post-transfection, the expression levels of HBsAg and EGFP were detected by using immunofluorescent staining (A), Western blotting (B), and Northern blotting (C).

p(3A)SAg-luc+ was replaced by pHBV3.6-luc+, which contained the whole HBV genome, similar results were observed (Fig. 2A). In addition, when the HBsAg-3 shRNA was expressed from the pHsU6 expression vector, which contained human RNA polymerase-III U6 RNA gene promoter, it also exerted the same inhibition effect on pHBV3.6-luc+-induced HBsAg expression (Fig. 2B). Moreover, the inhibition effect of HBsAg-3 shRNA was highly sequence specific because one or two nucleotide mutations would completely abolish its activity (Fig. 2C). Furthermore, the inhibition effect of HBsAg-3 shRNA was mechanically RNAi-directed because the pP_{HBsAg}-luc+-induced firefly luciferase production in which the expression of luciferase protein was under the control of HBsAg promoter was totally not affected by HBsAg-3 shRNA (data not shown). These results indicate that the selected HBsAg shRNAs, especially the HBsAg-3, 7, 9, and 10, could efficiently inhibit the HBsAg expression.

In vitro inhibition of HBsAg expression by HBsAg-3, 7, 9, and 10 shRNAs

To analyze in detail the inhibition effects of HBsAg-3, 7, 9, and 10 shRNAs on HBsAg expression, Huh7 cells were cotransfected with 0.5:1.5 μg p(3A)SAg-EGFP (or pHBV3.6-EGFP) and pSUPER-HBsAg-3 (or 7, 9, and 10) expression vectors by Lipofectamine 2000. At 60 h post-transfection, the expression levels of HBsAg in the transfected cells were detected by immunofluorescent staining. Both the p(3A)SAg-EGFP and pHBV3.6-EGFP expression vectors containing pEGFP-N1 expression cassette could be used as a marker to label the p(3A)SAg-EGFP- and pHBV3.6-EGFP-transfected cells. As the results shown in Fig. 3A, the HBsAg-3, 7, 9, and 10 shRNAs induced a complete inhibition of the HBsAg protein production but had no effect on EGFP expression, while the control vector and HBsAg-1 shRNA were totally ineffective on both the HBsAg and EGFP expression.

To further investigate the mechanism of the HBsAg-3, 7, 9, and 10 shRNAs mediated in vitro inhibition of HBsAg protein production, Huh7 cells were cotransfected with 0.5:1.5 μg of p(3A)SAg-EGFP (or pHBV3.6-EGFP) and pSUPER-HBsAg-3 (or 7, 9, and 10) expression vectors by Lipofectamine 2000. At 60 h post-transfection, total protein extracts and RNAs were isolated from transfected cells and analyzed by Western and Northern blotting, respectively. As shown in Fig. 3B, the HBsAg-3, 7, 9, and 10 shRNAs induced a strong inhibition of HBsAg protein production, but control vector and HBsAg-1 shRNA exhibited no inhibition on HBsAg protein production. Northern blot analysis of HBsAg expression, including p(3A)SAg-EGFP-induced 2.4- and 2.1-kb transcripts or pHBV3.6-EGFP-induced 3.5-, 2.4-, and 2.1-kb transcripts, revealed that the transcripts were completely inhibited by HBsAg-3, 7, 9, and 10 shRNAs, but not for the control vector and HBsAg-1 shRNA (Fig. 3C). These results indicate that in vitro inhibition of HBsAg expression by HBsAg-3, 7, 9, and 10 shRNAs is mediated by RNAi mechanism.

In vivo inhibition of HBsAg expression by HBsAg-3, 7, 9, and 10 shRNAs

Previously, we have reported a mouse HBV infection model by hydrodynamics-based transfection of viral

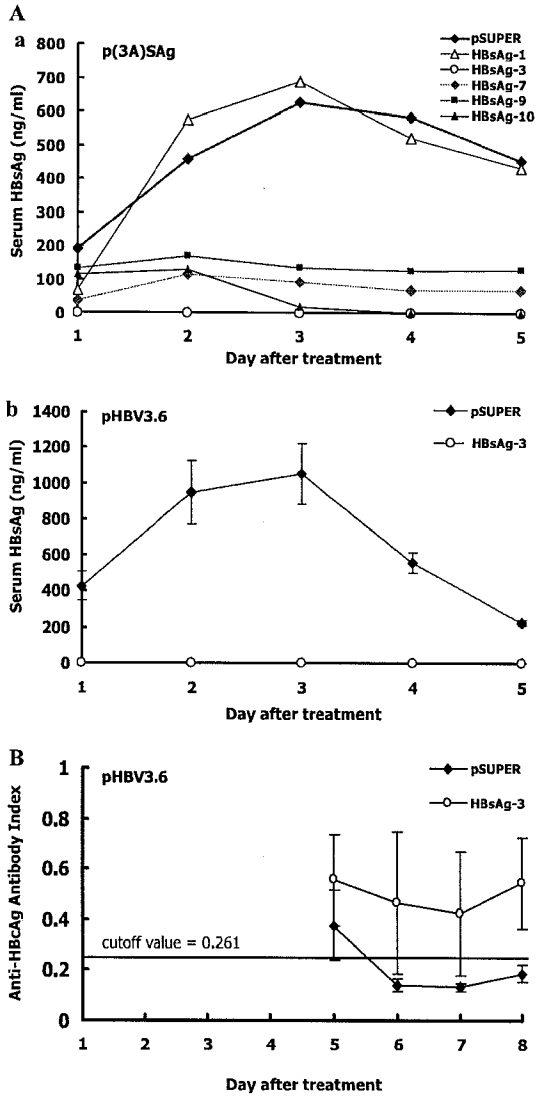


Fig. 4. (A) Inhibition effects of HBsAg-3, 7, 9, and 10 shRNAs on HBsAg expression in mice. Groups of six BALB/c mice were cotransfected intravenously with 2:10 μg p(3A)SAg and pSUPER-HBsAg-3, 7, 9, or 10 (a); or 5:15 μg pHBV3.6 and pSUPER-HBsAg-3 (b), by hydrodynamics-based transfection. The expression levels of HBsAg in sera were determined by HBsAg ELISA kit at every 24 h post-transfection. (B) Inhibition effects of HBsAg-3 shRNA on HBcAg expression in mice. Groups of six BALB/c mice were cotransfected intravenously with 5:15 μg pHBV3.6 and pSUPER-HBsAg-3 by hydrodynamics-based transfection. The expression levels of HBcAg in sera were determined indirectly by anti-HBcAg antibody assay. The cutoff value of anti-HBcAg antibody assay is 0.261 (1:5 dilution). The values below the cutoff value were defined as anti-HBcAg antibody positive.

DNA [46]. After hydrodynamic injection of pHBV3.6 expression vector, HBV genome was synthesized in the liver and HBV-DNA, as well as HBsAg and HBeAg were secreted into the blood. Anti-HBV antibody responses including anti-HBsAg and anti-HBcAg were developed afterward. Therefore, the inhibition effects of four selected HBsAg-3, 7, 9, and 10 shRNAs were evaluated on this in vivo model. When BALB/c mice were cotransfected intravenously with 2:10 μg p(3A)SAg and pSUPER-HBsAg-3 (or 7, 9, and 10) expression vectors by hydrodynamics-based transfection. The expression levels of HBsAg in sera were determined by ELISA kit at every 24 h post-transfection. As the results shown in Fig. 4A, the expression levels of HBsAg in sera were inhibited to different degrees by HBsAg-3, 7, 9, and 10 shRNAs. Consistent with the results of the in vitro cell culture experiments, the HBsAg-3 shRNA completely inhibited the serum HBsAg protein production, but control vector and HBsAg-1 shRNA did not exert any inhibition effect. This complete inhibition by HBsAg-3 shRNA was further demonstrated on whole HBV (pHBV3.6)-induced HBsAg protein production (Fig. 4A). In addition, the anti-HBcAg antibody response induced by pHBV3.6-expressed HBcAg was also completely inhibited by HBsAg-3 shRNA (Fig. 4B).

In vivo inhibition of HBV transcription and replication by HBsAg-3 shRNA

To fully analyze the inhibition effect of HBsAg-3 shRNA on HBV infection, the in vivo inhibition effects of HBsAg-3 shRNA on pHBV3.6- and pHBV3.6-EGFP-induced HBV transcription and replication were elucidated. BALB/c mice were cotransfected intravenously with 5:15 μg pHBV3.6-EGFP and pSUPER-HBsAg-3 expression vectors by hydrodynamics-based transfection. At 2 days post-transfection, the livers were removed and the expression levels of HBsAg were analyzed by Western blotting. The pHBV3.6-EGFP-induced HBsAg protein production on livers was strongly inhibited by HBsAg-3 shRNA, but control vector had totally no effect in comparison with mock (Fig. 5A). To examine this inhibition effect directly, total RNAs isolated from livers were analyzed for the intermediated transcripts of HBV by Northern blotting. As shown in Fig. 5B, three HBV RNA transcripts including 3.5-, 2.4-, and 2.1-kb were decreased to a very low level in compared to the vector controls and mock.

To test whether HBsAg-3 shRNA could also inhibit HBV replication in mouse HBV model induced by hydrodynamics-based transfection of HBV-DNA, a cotransfec-

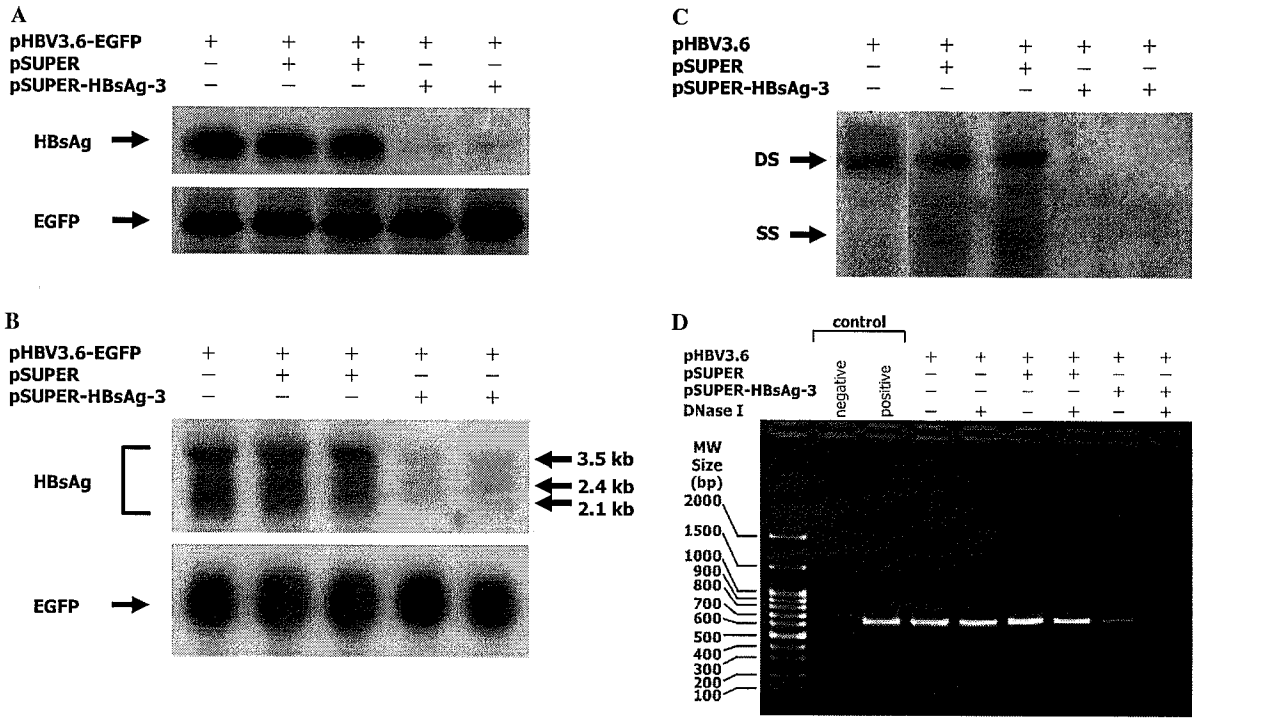


Fig. 5. Inhibition effects of HBsAg-3 shRNA on HBV transcription and replication in mice. Groups of BALB/c mice were cotransfected intravenously with 5:15 μg pHBV3.6-EGFP and pSUPER-HBsAg-3 by hydrodynamics-based transfection. At 2 days post-transfection, the livers were removed and the expression levels of HBsAg were analyzed by Western blotting (A) and Northern blotting (B). Northern blot analysis of HBV transcripts (3.5, 2.4, and 2.1 kb) was performed with EGFP gene as RNA loading controls. Inhibition effects of HBsAg-3 shRNA on HBV DNA production in mouse liver tissue (C) and serum (D). The double stranded (DS) or single stranded (SS) HBV DNA replicative forms were indicated. Mouse serum was collected at 2 days post-injection from the above experiment. DNA was purified from mouse serum before and after treatment with 20 U DNase I to remove retaining plasmid and used as template to amplify the pre-S region of HBV genome. The specific PCR product of 654 bp was detected in mouse serum. The smaller band (approximate 40 bp) appeared was either primers or primer-dimers. MW, 100 bp DNA ladder. Positive control represents the PCR in the presence of 5 pg of pHBV3.6 plasmid.

tion experiment was conducted. BALB/c mice were cotransfected intravenously with 5:15 μg pHBV3.6 and pSUPER-HBsAg-3 expression vectors by hydrodynamics-based transfection. At 2 days post-transfection, cytoplasmic DNAs were isolated from livers and analyzed by Southern blotting. The double stranded (DS) or single stranded (SS) HBV-DNA replicative forms were inhibited completely (Fig. 5C). In addition, the levels of HBV-DNA in sera were also measured in BALB/c mice after pHBV3.6 and pSUPER-HBsAg-3 cotransfection. The sera were treated without or with DNAse I to eliminate pHBV3.6 plasmid DNA, the HBV particle synthesized and released into sera after pHBV3.6 transfection was detected by HBV-specific PCR analysis. As shown in Fig. 5D, HBV-DNA in sera was also inhibited completely by HBsAg-3 shRNA.

Therapeutic effect of HBsAg-3 shRNA in post-treatment protocol and transgenic mice model

To investigate whether HBsAg-3 shRNA could therapeutically inhibit HBsAg expression in the in vivo mouse HBV model induced by hydrodynamics-based transfection of HBV-DNA, a post-treatment protocol was conducted. BALB/c mice were transfected intravenously with 5 μg p(3A)SAg to induce HBsAg protein production by hydrodynamics-based transfection. At 2 days later, 25 μg pSUPER-HBsAg-3 was then transfected intravenously again. The expression levels of HBsAg in sera were measured by HBsAg ELISA kit. As the results shown in Fig. 6A, the HBsAg was inhibited about 18% at day one, 60% at day two–four, and 75% at day five after pSUPER-HBsAg-3 treatment. This therapeutic effect was also found for pHBV3.6-induced HBsAg expression. Inhibition of 15% and 90% of HBsAg protein production was found at day one and day two after treatment, respectively (Fig. 6A). In addition, anti-HBcAg antibody induction was also blocked by HBsAg-3 shRNA therapeutically in pHBV3.6-induced HBcAg protein production (Fig. 6B).

To further test this therapeutic inhibition, the effect of HBsAg-3 shRNA on transgenic mice that constitutively expressed HBsAg in livers was examined. HBsAg transgenic FVB/N mice were transfected intravenously with 25 μg of pSUPER-HBsAg-3 by hydrodynamics-based transfection. At 3 days post-transfection, the livers were removed and the expression levels of HBsAg were analyzed by immunohistochemical staining, Western and Northern blot analyses. As shown in Fig. 7A, the expression of HBsAg on hepatocytes of transgenic mice was strongly inhibited after hydrodynamics-based transfection of pSUPER-HBsAg-3. While the control vector pSUPER was ineffective. The Western blot analysis further confirmed the inhibition of HBsAg production in transgenic mice livers by expression of the HBsAg-3 shRNA (Fig. 7B). In addition, the Northern blot analysis showed that HBsAg-3 shRNA induced strong reduction of HBsAg mRNA, indicating that the therapeutic inhibition of HBsAg expression by HBsAg-3 shRNA was mediated by RNAi mechanism (Fig. 7C).

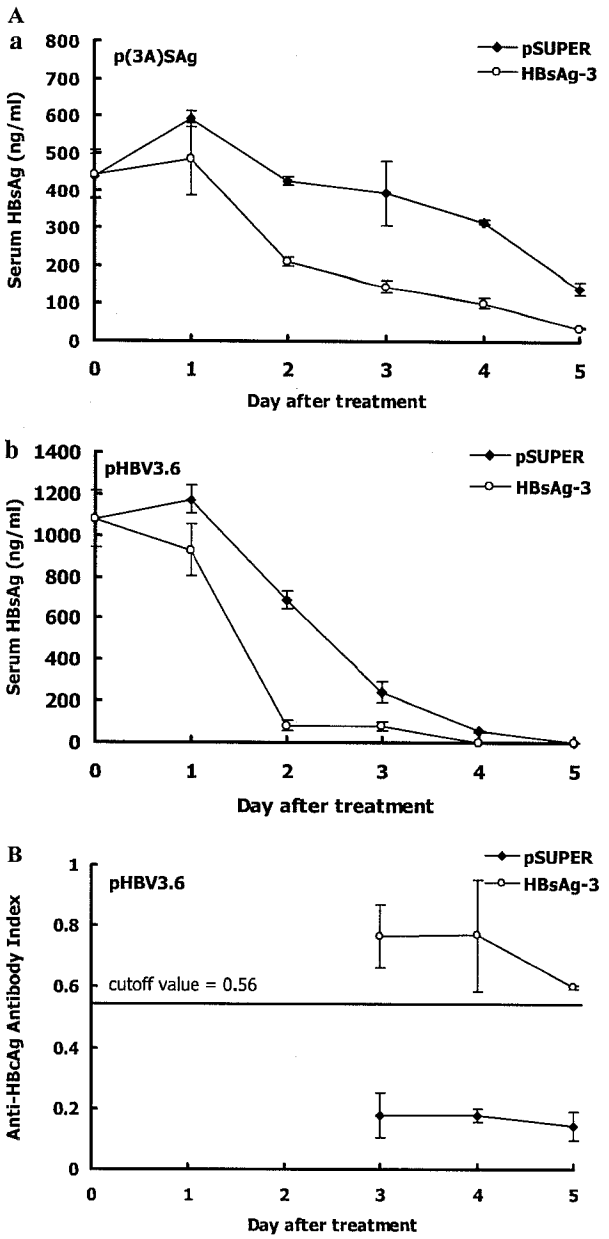


Fig. 6. Inhibition effects of HBsAg-3 shRNA on HBsAg and HBcAg expression in mice by post-treatment protocol. (A) Inhibition effects of HBsAg-3 shRNA on HBsAg expressed mice. Groups of six BALB/c mice were transfected intravenously with 5 μg p(3A)SAg (a); or pHBV3.6 (b), by hydrodynamics-based transfection. At two days later, 25 μg of pSUPER-HBsAg-3 was given again by hydrodynamics-based transfection. The expression level of HBsAg in serum was detected by ELISA kit. The day of second injection was defined as day 0. (B) Inhibition effects of HBsAg-3 shRNA on HBcAg expressed mice. Groups of six BALB/c mice were transfected intravenously with 5 μg pHBV3.6 by hydrodynamics-based transfection. At two days later, 25 μg of pSUPER-HBsAg-3 was given again by hydrodynamics-based transfection. The expression level of HBcAg in serum was measured indirectly by anti-HBcAg antibody assay. The cutoff value of anti-HBcAg antibody assay is 0.56. The values below the cutoff value were defined as anti-HBcAg antibody positive.

Based on the above results, we conclude that HBsAg-3 shRNA could effectively inhibit the HBsAg expression on a therapeutic basis.

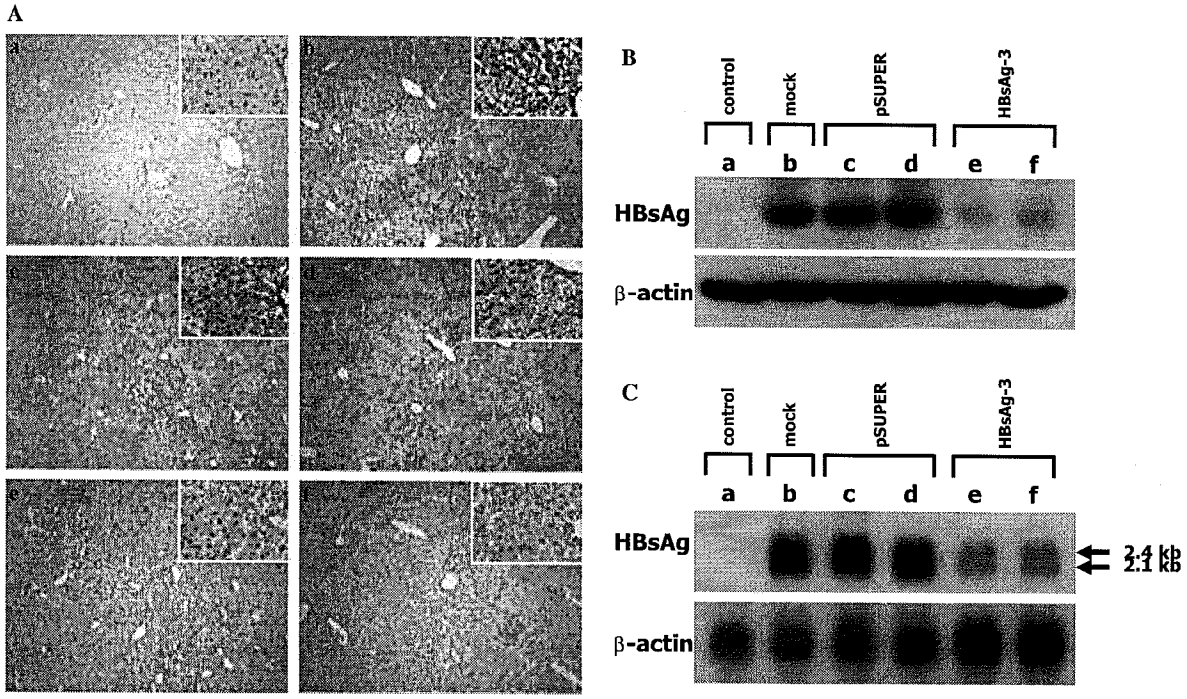


Fig. 7. Inhibition effect of HBsAg-3 shRNA on HBsAg expression in HBsAg transgenic mice. Groups of HBsAg transgenic mice were transfected intravenously with 25 μg pSUPER-HBsAg-3 by hydrodynamics-based transfection. The liver tissues were collected at 3 days post-injection for analysis of the HBsAg expression. (A) Immunohistochemical staining of HBsAg with anti-HBsAg antibody: (a) non-transgenic FVB/N mouse as a negative control; (b) non-treated HBsAg transgenic mouse as a positive control; (c,d) two representative pSUPER treated HBsAg transgenic mice; (e,f) two representative pSUPER-HBsAg-3 treated HBsAg transgenic mice. Original magnification: 40× (insets: 100×). (B) Western blot analysis of HBsAg in total liver protein extracts. (C) Northern blot analysis of 2.4- and 2.1-kb HBsAg mRNAs in total liver RNAs. β-Actin serves as an internal control.

Discussion

Chronic HBV infection is a major health problem worldwide, not only for the manifestation of chronic hepatitis and cirrhosis, but also for the increasing risk to become HCC. It has been reported that the late non-replication phase of chronic HBV infection exclusively associated with the HBsAg expression, and almost all the HBV-associated HCCs harbored chromosomally integrated viral DNAs [48,49]. In addition, a large number of mutations and deletions in HBV genome have been reported during the persistent virus infection [50–52]. Although HBx has been implicated in the process of HCC, the viral DNA-integrated genome is commonly found to contain a partial or complete deletion of the HBx gene [53–57]. Moreover, the development of the HCC is preceded by chronic liver injury and inflammation. Studies on HBsAg transgenic mice revealed that a severe, prolonged hepatocellular injury, regenerative hyperplasia, and a secondary inflammation preceded the HCC [58–60]. Therefore, the tactics for treating chronic HBV infection should not be restricted to the inhibition of the virus replication, the suppression of the HBsAg expression should also be considered.

Previous studies have demonstrated that either DNA vector-expressed or synthetic siRNAs could inhibit viral DNA-induced HBV replication and expression by directly targeting the HBcAg, HBsAg or HBx gene [25,26,43,44]. In this study, we specially focused on the HBsAg gene including pre-S1, pre-S2, and in particular S regions by selectively targeting the sequences that have not been shown any therapeutic intervention-induced mutations. By using highly effective shRNA expression system, pSUPER [9], an extremely potent and highly sequence-specific HBsAg-3 shRNA was identified that not only inhibited the HBsAg expression but also suppressed the HBV replication in either hydrodynamics-based transfection experiments or HBsAg transgenic mice model. The hydrodynamics-based transfection of the pHBV3.6 into mouse liver resulted in viral DNA replication, antigen, and antibody responses is considered to mimic acute HBV infection [46]. The HBsAg transgenic mouse that constitutively expressed HBsAg in the liver and secreted surface antigen into the serum is regarded as a chronic HBV infection at the non-replicative resident integration stage.

Many studies including this report have found that the distinct RNAi targeting sequences on HBsAg gene exhibited different efficacies on inhibition of the viral DNA replication and gene expression [25,26,43,44]. In addition, the highest inhibition effects induced by the different siRNAs targeted on HBcAg or HBx gene have also been reported [25,26]. The particular advantage of using RNAi-based therapeutic strategy over conventional drugs such as Lamivudine [37] is that siRNA mediated inhibition of gene

expression does not require any viral DNA replication. Therefore, to efficiently apply the RNAi-based therapeutic strategy for treatment of the HBV-induced diseases in particular the chronic HBV infection, it is important to use a combination of various highly effective siRNAs against including HBV P, HBcAg, HBsAg, and HBx genes to achieve a complete inhibition of virus replication and gene expression. To advance toward this goal, further investigations to prescribe the best combination of the effective siRNAs are warranted.

Acknowledgment

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Exhibit B

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Inhibition of hepatitis B virus in mice by RNA interference

Anton P McCaffrey[1], Hiroyuki Nakai[1,4], Kusum Pandey[1,4], Zan Huang[1,4], Felix H Salazar[2], Hui Xu[1], Stefan F Wieland[3], Patricia L Marion[2] & Mark A Kay[1]

Hepatitis B virus (HBV) infection substantially increases the risk of chronic liver disease and hepatocellular carcinoma in humans. RNA interference (RNAi) of virus-specific genes has emerged as a potential antiviral mechanism. Here we show that RNAi can be applied to inhibit production of HBV replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with an HBV plasmid. Cotransfection with plasmids expressing short hairpin RNAs (shRNAs) homologous to HBV mRNAs induced an RNAi response. Northern and Southern analyses of mouse liver RNA and DNA showed substantially reduced levels of HBV RNAs and replicated HBV genomes upon RNAi treatment. Secreted HBV surface antigen (HBsAg) was reduced by 94.2% in cell culture and 84.5% in mouse serum, whereas immunohistochemical detection of HBV core antigen (HBcAg) revealed >99% reduction in stained hepatocytes upon RNAi treatment. Thus, RNAi effectively inhibited replication initiation in cultured cells and mammalian liver, showing that such an approach could be useful in the treatment of viral diseases.

RNA interference is a process during which cytoplasmic long double-stranded RNAs (dsRNAs) produced by viral infection, by transposons or by introduced transgenes are targeted for inactivation[1]. These long dsRNAs are processed into 21- to 23-nucleotide (nt) guide RNA duplexes by an RNase called Dicer[2] and are further incorporated into an RNA-induced silencing complex (RISC)[3]. The RISC complex uses the guide RNAs to identify homologous RNAs in the cell and proceeds to cleave them. Synthetic small interfering RNAs (siRNAs)[4] and shRNAs transcribed *in vivo* from DNA templates trigger specific silencing of genes when transfected into cultured cells[5]. Several groups have inhibited replication of viruses in culture using RNAi[6–12], including inhibition of HBV in cultured cells[13]. However, there are no reports of *in vivo* viral inhibition by RNAi in mammals. Thus, it remains unclear how RNAi might function in the context of the functional innate and adaptive immune systems present in whole animals. Here we examine the antiviral effect of shRNAs targeting HBV in mice.

Recently, we showed that synthetic siRNAs as well as shRNAs transcribed *in vivo* from DNA templates were potent inhibitors of gene expression in adult mice. Furthermore, we showed that a sequence from the hepatitis C virus (HCV), fused to a reporter gene, could be targeted by RNAi, suggesting the possibility of using RNAi as a therapeutic tool[14]. However, only a portion of the viral genome was present, and viral replication did not occur in that particular HCV model system. In contrast, many of the steps in the viral replication cycle of HBV occur in mice after transfection with plasmids containing the HBV genome[15]. This model system offers the opportunity to test the ability of RNAi to inhibit replication of a virus in a small animal model of human disease. Here we demonstrate that expressed shRNAs targeting HBV mRNAs inhibited the steps in HBV replication that occur in cultured cells and in immunocompetent and immunodeficient mice.

RESULTS

Selection of RNAi target sites

Seven RNAi target sequences were chosen on the basis of their conservation among the major HBV genotypes[16]. In some cases, these sequences targeted overlapping reading frames of the virus such that multiple viral RNAs would be inhibited by one shRNA (**Fig. 1a**). Each shRNA targets the pregenomic RNA serving as the template for HBV genomic replication as well as the mRNA for the core antigen and the polymerase. shRNAs HBVU6no.1, HBVU6no.2, HBVU6no.3 and HBVU6no.4 also target the HBV S-antigen mRNAs. HBVU6no.5 also targets the X region and its transcript, whereas HBVU6no.6 and HBVU6no.7 target the pregenomic RNA in the overlap region encoding the core antigen and the polymerase. A schematic of the U6 shRNA expression cassette (**Fig. 1b**) and, as an example, the predicted folding of HBVU6no.2 (**Fig. 1c**) are shown.

HbsAg expression in cell culture is inhibited by HBV RNAi

To test whether RNAi could inhibit HBV in cell culture, a cotransfection assay was conducted. In each experiment three plasmids were cotransfected into cultured HuH-7 cells (a human hepatoma cell line): (i) 4 μg of the plasmid pTHBV2 (ref. 17) containing the HBV genome with some sequences duplicated to allow complete expression of all genes; (ii) 5 μg of a U6 shRNA expression vector, or either empty vector that does not express shRNAs or an shRNA vector targeting HCV (negative control RNAi) as negative controls; and (iii) 5 μg of a plasmid

[1]Departments of Pediatrics and Genetics, Stanford University School of Medicine, 300 Pasteur Drive, Room G305, Stanford, California, USA. [2]Hepadnavirus Testing, Inc., 831H Sierra Vista Ave., Mountain View, California, USA. [3]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA. [4]These authors contributed equally to this work. Correspondence should be addressed to M.A.K. (markay@stanford.edu).

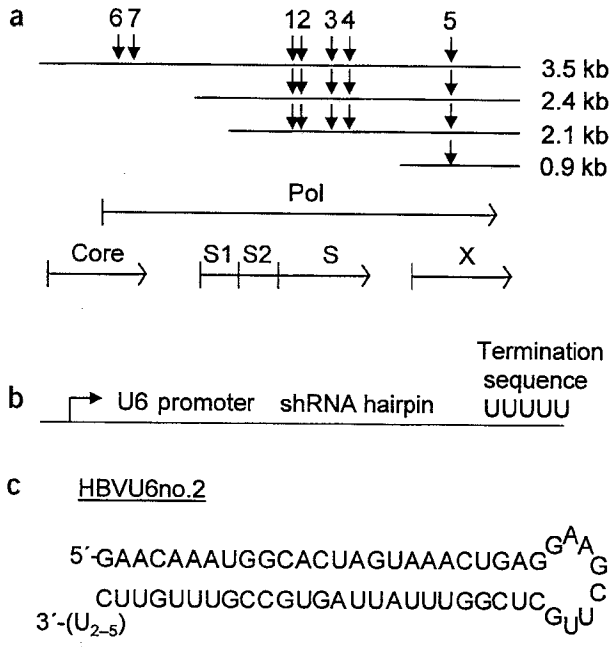

Figure 1 Target site and vector information. (**a**) Location of RNAi target sites. Downward arrows indicate the location of RNAi target sites within the four HBV transcripts. The 3.5-kb transcript is the pregenomic RNA that serves as the template for HBV viral DNA replication. The HBV open reading frames are shown below aligned with the HBV mRNAs. Pol, polymerase; Core, HBcAg; S1, large pre-surface antigen; S2, middle pre-surface antigen; S, HBsAg; X, X gene. The numbers above the arrows indicate the shRNA that targets that site. (**b**) Schematic of U6 promoter constructs. Transcription ends at a string of 5 Us. (**c**) Predicted folding of HBVU6no.2.

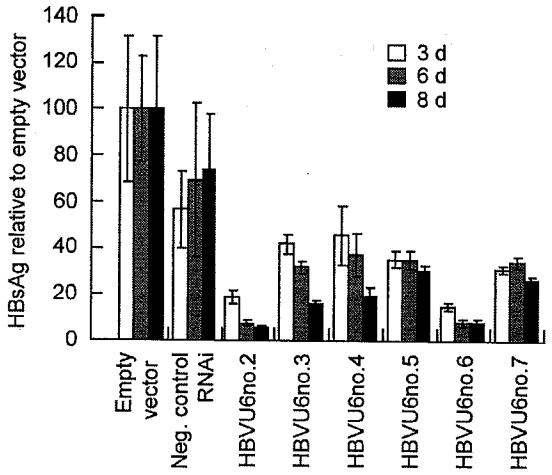

Figure 2 HBsAg measurements in medium of shRNA-treated cultured cells. HBsAg levels in culture medium are significantly reduced by treatment with HBV RNAi. Standard errors are shown. Observed reductions in HBsAg did not result from poor transfection, nonspecific inhibition of translation or toxicity, because the average hAAT levels for experimental groups were not reduced compared to the control group.

(pThAAT) that expresses the secreted protein human α1-antitrypsin (hAAT)[18]. Transfection with pTHBV2 initiated an HBV replication cycle, resulting in production of replicated HBV genomes as well as viral mRNAs and proteins (including HBsAg and HbcAg). ELISA measurements of secreted hAAT[19] monitored for transfection efficiency and nonspecific translational inhibition or toxicity. Average hAAT concentrations in culture medium at days 3 and 7 were similar in all groups.

On days 3, 6 and 8, HBsAg levels in the medium were measured (Fig. 2). With the exception of HBVU6no.1 (data not shown), treatment with each of the shRNA expression plasmids reduced the amount of HBsAg compared with the untreated control group, in three independent experiments. Treatment with HBVU6no.2 and HBVU6no.6 gave the greatest reduction in HBsAg (94.2 ± 0.59% ($P = 0.0003$) and 91.5 ± 1.4% ($P = 0.0003$) on day 8, respectively), compared with the empty-vector control. These results demonstrate that RNAi can substantially inhibit HBV viral protein expression and therefore could ultimately suppress viral replication in cultured cells. The best inhibitors (HBVU6no.2 and HBVU6no.6) were studied further in mice.

HBV RNAs in mice are reduced by HBV shRNAs

Northern hybridization analysis was conducted to determine if treatment with HBVU6no.2 and HBVU6no.6 resulted in a reduction in the amount of HBV RNAs present in immunocompetent (C57BL/6J, Fig. 3a) or immunocompromised NOD/LtSz-*Prkdc*^{scid}/J (NOD SCID, Fig. 3b) mice. DNAs were transfected into mouse liver by hydrodynamic transfection[20,21], a method that results in gene transfer into 5–40% of mouse hepatocytes[14]. Mice were transfected with 12 μg of pTHBV2, 5 μg of pThAAT and 5 μg of a control plasmid or shRNA expression plasmid. At day 7, total liver RNA was analyzed by northern hybridization. No bands were detected in total RNA from a naive mouse that was not transfected with pTHBV2 (**Fig. 3a**, lane 1). In total RNA samples from mice that received pTHBV2 (**Fig. 3a**, lanes 2–9; **Fig. 3b**, lanes 1–8), a 3.5-kilobase (kb) pregenomic RNA encoding the viral core and polymerase proteins was observed. RNAs of 2.4 kb and 2.1 kb encoding the viral envelope proteins were also observed; they comigrated in most lanes, although in a sample from an HBV transgenic mouse[17], these two bands were barely resolved (**Fig. 3a**, lane 16). Consistent with a previous report[15], the transcript encoding the X protein was not observed. Northern blotted membranes were also probed for transcript of the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as a loading control.

Substantial amounts of HBV RNAs were observed in total RNA from mice that did not receive an RNAi plasmid (No RNAi group, **Fig. 3a**, lanes 2 and 3; **Fig. 3b**, lanes 1 and 2). Mice that received a negative control shRNA plasmid (Neg. control RNAi) targeting HCV also showed substantial amounts of HBV RNAs (**Fig. 3a**, lanes 4 and 5; **Fig. 3b**, lanes 3 and 4). Compared with mice from the No RNAi group, immunocompetent and immunocompromised mice treated with HBVU6no.2 had 77% and 92% less HBV RNA, respectively, after normalization to the GAPDH endogenous transcript (**Fig. 3a**, lanes 6 and 7, **Fig. 3b**, lanes 5 and 6). Compared with the control No RNAi group, HBVU6no.6-treated immunocompetent (**Fig. 3a**, lanes 8 and 9) and immunocompromised (**Fig. 3a**, lanes 7 and 8) mice had reductions in HBV RNA of 31% and 58%, respectively. Thus, treatment with HBV shRNAs reduces HBV RNA transcripts.

HBVU6no.2 targets all three viral RNAs; however, HBVU6no.6 targets only the 3.5-kb transcript and should not reduce the levels of the 2.1- and 2.4-kb RNAs. HBVU6no.6 reduces all viral transcripts at the dose used, suggesting that some mechanism other than RNAi must be operating. This effect is observed in immunocompromised mice, suggesting that it does not result from an antigen-dependent immune response. The implications of these results are discussed later.

Replicated HBV genomes were reduced by HBV RNAi

Hydrodynamic transfection with pTHBV2 results in the production of HBV DNA-replicative intermediates in mice. Single-stranded (ss) and double-stranded (ds) replicative intermediates were observed (Fig. 4a, lane 17) for a replicative intermediate marker derived from a cell line that stably expresses HBV DNAs. However, no closed circular DNA (cccDNA) was observed in a similar hydrodynamic HBV model[15], suggesting that the full viral replication cycle cannot be completed in mice. Analysis of our DNA samples also failed to detect cccDNA (Supplementary Fig. 1 online). Nonetheless, this model recapitulates most of the steps of HBV viral replication and thus serves as a good means for the evaluation of inhibitors of HBV replication. NOD SCID mice received 12 μg of pTHBV2 as well as 5 μg of one of the following: (i) no RNAi plasmid, (ii) an HCV shRNA expression plasmid (Neg., control RNAi), (iii) pHBVU6no.2 or (iv) pHBVU6no.6. Mice were killed at day 7 and total DNA extracted.

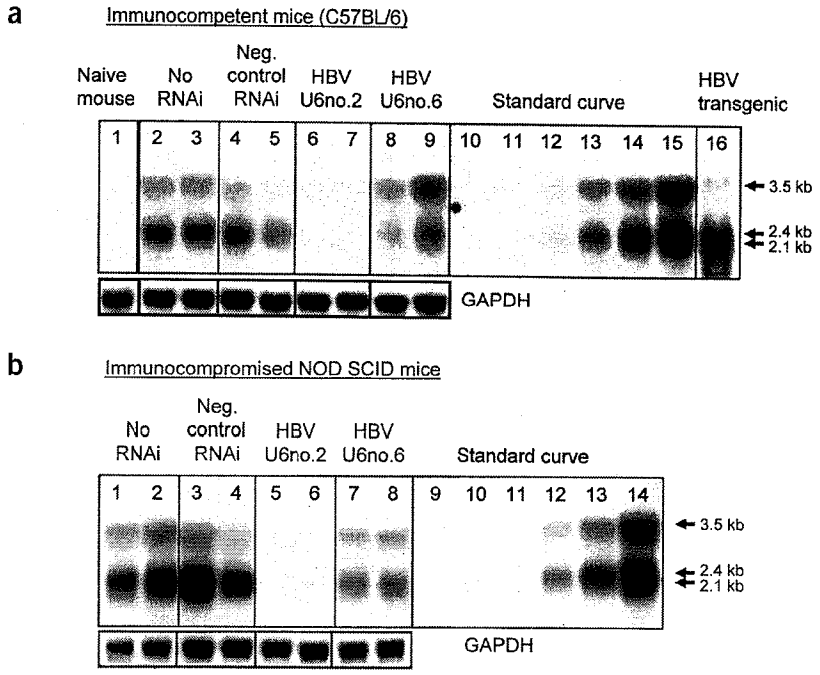

Figure 3 Northern hybridization analysis of HBV RNAs. (**a**) Samples from immunocompetent mice (C57BL/6). (**b**) Samples from immunocompromised NOD/LtSz-*Prkdc*^{scid}/J (NOD SCID) mice. Lane 1 in **a**, no bands are detected in RNA from a naive mouse (not transfected with pTHBV2). Lanes 2–9 in **a**, HBV pregenomic and HBsAg mRNAs are observed. Reported HBV RNA levels were normalized with the levels of the endogenous GAPDH transcript. Compared to mice from the No RNAi group (lanes 2 and 3 in **a**, lanes 1 and 2 in **b**), mice from the Neg. control RNAi group (lanes 4 and 5 in **a**, lanes 3 and 4 in **b**) had similar normalized amounts of HBV RNA. Mice that received HBVU6no.2 (lanes 6 and 7 in **a**, lanes 5 and 6 in **b**) had substantially reduced amounts of HBV RNA. Mice that received HBVU6no.7 also had reduced levels of both the 3.5-kb pregenomic RNA and the HBsAg mRNAs (lanes 8 and 9 in **a**, lanes 7 and 8 in **b**). Lane 16 in **a** shows total RNA from an HBV transgenic mouse. The HBV 3.5-kb pregenomic RNA as well as the 2.4- and 2.1-kb HBsAg mRNAs were observed. An endogenous GAPDH transcript controls for loading integrity of the RNA.

To determine definitively if treatment with HBVU6no.2 and HBVU6no.6 resulted in a reduction in replicated viral genome intermediates in the livers of mice, a modified Southern hybridization assay was conducted that detects replicated viral genomes (*Dpn*I insensitive) but not bacterially methylated input plasmid (*Dpn*I sensitive). Some DNA samples were not treated with *Dpn*I (Fig. 4a), allowing visualization of the input plasmid. The same DNA samples were run on a separate gel and the copy number per cell of the input plasmid, pTHBV2, was determined by Southern hybridization using a probe for the ampicillin resistance gene present on this plasmid (copy number listed in Fig. 4a). The same DNA samples were then treated with *Dpn*I to degrade input plasmid (Fig. 4b). Conditions that resulted in complete digestion of at least five copies per cell of input plasmid were used, which is sufficient to degrade all the bacterially methylated input DNA (Fig. 4b, lane 17). The amount of replicated DNA was quantified by phosphorimager analysis.

Replicated HBV genomic DNA was present in samples from mice in the control groups that did not receive an RNAi plasmid (Fig. 4a,b, lanes 5–7). Mice that received the negative control RNAi (Fig. 4a,b, lanes 8–10) had slightly less replicated DNA than the No RNAi group. DNA from HBVU6no.2-treated mice (Fig. 4a,b, lanes 11–13) had undetectable levels of replicated HBV genomes. HBVU6no.6-treated mice (Fig. 4a,b, lanes 14–16) had somewhat fewer replicated HBV DNA molecules. The Southern blotted membrane was stripped and reprobed for the endogenous mouse (agouti) gene, as a means of verifying that the observed difference in replicated HBV genomes did not result from variation of integrity of DNA samples loaded on the gels. Variation in the copy number of the HBV plasmid also did not account for the observed differences in replicated HBV genomes.

In contrast with the No RNAi group, ssHBV genome was the predominant molecular form in RNAi-treated mice (including the negative control RNAi). We cannot exclude the possibility that low levels of nuclease activity converted partially dsDNA to ssDNA. Nevertheless, RNAi directed against HBV results in substantial reduction in all of the steps of replication of HBV genomes that occur in immunocompromised mice. Because these experiments were conducted in NOD SCID mice, the observed reductions in HBV genomes did not result from cytotoxic T cell–mediated events. We carried out the same analysis using *Dpn*I-treated DNA from immunocompetent BALB/c mice and also observed substantial reductions in HBV genomes (data not shown). Thus in both immunodeficient and immunocompetent mice, treatment with HBV shRNAs resulted in substantial reduction in all of the HBV-replicative forms produced in mice.

HBsAg expression in mice is inhibited by HBV RNAi

The same three-plasmid cotransfection model was used to test RNAi inhibition of HBsAg production in mice. Yang *et al.* reported that upon hydrodynamic transfection with an HBV plasmid in immunocompetent mice, HBsAg-neutralizing antibodies were observed starting at day 7 (ref. 15). To avoid complications associated with neutralizing antibodies, experiments were carried out in NOD SCID mice lacking B and T lymphocytes. Serum HBsAg was measured at day 4 and day 7 (Fig. 5).

At day 4, serum HBsAg for the Neg. control RNAi group and the HBVRNAino.6 group was similar to that of the No RNAi group

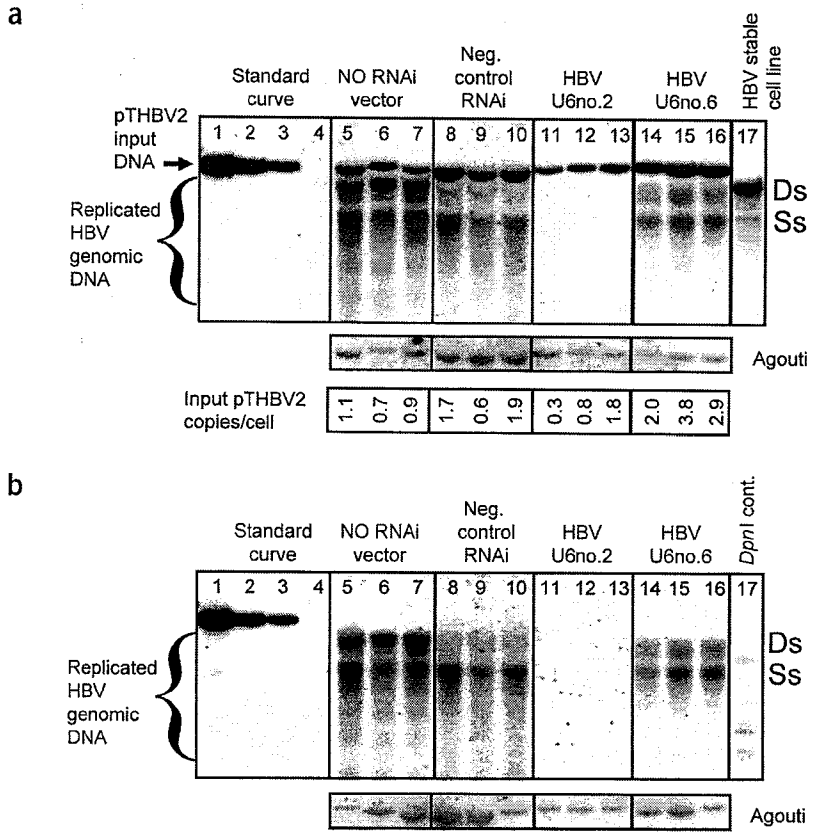

Figure 4 Southern hybridization analysis of HBV DNAs. (**a**,**b**) Southern hybridization analysis of HBV genomic DNA from mice that received (i) no RNAi plasmid, (ii) an HCV shRNA expression plasmid (Neg. control RNAi), (iii) pHBVU6no.2 or (iv) pHBVU6no.6. (**a**) Input ~7.3-kb plasmid is observed in total DNA samples that were treated only with *Sac*I but not with *Dpn*I. (**b**) To remove input plasmid DNA and leave only replicated HBV genomes, total DNA samples were treated with *Dpn*I together with *Sac*I. *Dpn*I cleaves the bacterially methylated input DNA (pTHBV2) in 34 locations but does not cleave unmethylated replicated HBV genomes. Total DNA samples from mice that received 12 μg of pTHBV2 and no RNAi plasmid (lanes 5–7 in **a** and **b**), an HCV shRNA Neg. control RNAi (lanes 8–10 in **a** and **b**), pHBVU6no.2 (lanes 11–13 in **a** and **b**) or pHBVU6no.6 (lanes 14–16 **a** and **b**) are shown. Replicated HBV genomes were undetectable in DNA samples from mice that received HBVU6no.2 and were substantially reduced in mice that received pHBVU6no.6. A standard curve was generated by spiking 25, 5, 1 or 0 copies/cell of *Sac*I-digested pTHBV2 into 10 μg of total mouse DNA (lanes 1–4 in **a** and **b**, respectively). To ensure that the *Dpn*I digestion conditions used to degrade the input DNA were sufficient, 5 copies/cell of pTHBV2 were spiked into 10 μg of total mouse DNA (lane 17 in **b**) and digested in the same way. Replicated HBV ds and ssDNA is observed in DNase I-resistant cytoplasmic DNA extracted from 2.2.15 cells that stably express HBV genomic DNAs[24].

($P = 0.793$ and $P = 0.971$, respectively). In contrast, serum HBsAg of HBVU6no.2-treated mice at day 4 was reduced by 88% (± 2.2%; $P = 0.0119$) compared with the No RNAi group. Compared to the No RNAi group, at day 7, serum HBsAg for the Neg. control RNAi group was statistically similar ($P = 0.194$), whereas HBsAg for the HBVU6no.2 and HBVU6no.6 groups was reduced by 85% (± 3.3%; $P < 0.0001$) and 47% (± 9.3%; $P = 0.0088$), respectively.

At days 4 and 7, serum hAAT was measured to ensure that transfection efficiencies were similar and to exclude the possibility of nonspecific translational arrest resulting from treatment with shRNAs. Average hAAT amounts varied by no more than twofold and declined by similar amounts for all groups between day 4 and day 7. These results demonstrated that HBVU6no.2 and pHBVU6no.6 shRNAs substantially reduce serum HBsAg in mice. Furthermore, the decrease in serum HBsAg was not a result of nonspecific translational arrest resulting from expression of short duplex RNAs, because hAAT levels were similar in all groups. The observation that HBVU6no.6 reduces serum HBsAg was unexpected, because it does not target the HBsAg mRNA; however, this observation correlates well with the observed reduction in HBsAg mRNA upon treatment with HBVU6no.6.

HBcAg expression in mice is inhibited by RNAi

HBcAg, the nucleocapsid protein, is synthesized in infected cells and is required for HBV viral replication. At day 7, paraffin-fixed liver sections were prepared from mice transfected with 5 μg HBVU6no.2, HBVU6no.6 or empty vector as well as 4 μg pTHBV2. Consistent with the expected transfection efficiency, 5.2 ± 1.1% of cells stained for HBcAg in tissue sections from mice that received the empty vector and pTHBV2 (Fig. 6a,b). Liver sections from mice receiving HBVU6no.2 had significantly reduced numbers of HBcAg-stained cells (reduced by 99.7 ± 0.3%; $P = 0.0011$). Most fields contained no stained cells (Fig. 6c), although there were rare hepatocytes with lightly stained nuclei (Fig. 6d). The number of stained hepatocytes in sections from HBVU6no.6-treated mice was reduced by 94 ± 1.9% ($P = 0.0014$) (Fig. 6e). No staining was seen in sections from mice that did not receive pTHBV2 (Fig. 6f). These results demonstrate that HBV RNAi can inhibit the production of HBV proteins.

DISCUSSION

There has been considerable interest in the use of RNAi therapeutics to treat a wide range of diseases. Recent reports that RNAi can inhibit viral replication in cell culture support this notion[6–13]. Previously, we demonstrated RNAi was functional in mice and could target sequences from HCV[14]. Recently, similar methods were used to inhibit the production of Fas receptor in mice. Fas siRNA treatment protected mice from fulminant hepatitis induced by injecting agonistic Fas-specific antibody[22]. These reports demonstrate the RNAi can be used therapeutically in mammals. However, to date there has been no report of RNAi inhibition of a virus in mammals. Here, we show that RNAi can inhibit all the steps of HBV replication that occur in cell culture and in mice. Thus, it may be possible to use shRNAs directed against the viral or host genes, to inhibit viral replication, and inhibit or reverse the fibrosis associated with the development of liver cirrhosis.

Four separate lines of evidence establish that RNAi substantially inhibited HBV in mice: (i) RNAi expression significantly reduced secreted HBsAg in culture medium and in mouse serum; (ii) HBV RNAs were substantially reduced in mouse liver; (iii) HBV genomic DNA was reduced to undetectable levels in mouse liver; and (iv) the

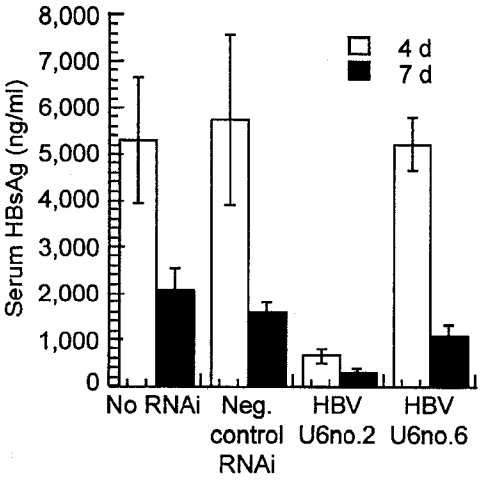

Figure 5 Serum HBsAg levels in shRNA-treated mice. HBsAg levels in NOD SCID mouse sera are significantly reduced after treatment with the shRNA expression plasmids HBVU6no.2 and HBVU6no.6 (error bars indicate standard errors).

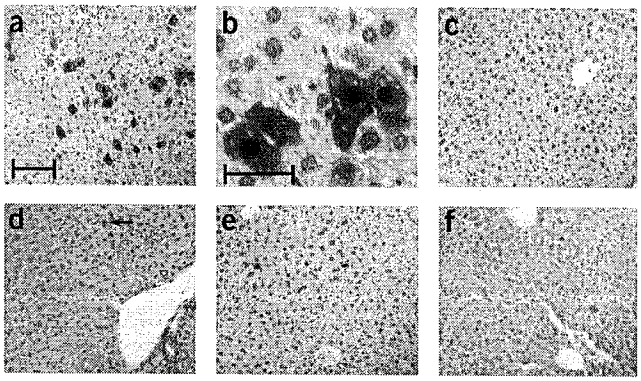

Figure 6 Immunohistochemical staining for HBcAg in liver sections. (**a–f**) Treatment with HBVU6no.2 or HBVU6no.6 reduces the number of cells that stain for HBcAg. Immunohistochemical staining for HBcAg was done on tissue sections from three animals per group. Representative sections are shown (four sections per animal were stained and counted). Paraffin-embedded sections from animals that received 4 μg pTHBV2, 3 μg pThAAT and 5 μg of indicated plasmid. (**a**) Empty vector (10× magnification, scale bar 100 μm). (**b**) Empty vector (40× magnification, scale bar 10 μm). (**c**) HBVU6no.2; no stained cells were observed in most fields. (**d**) HBVU6no.2; faintly stained cell indicated by an arrow. (**e**) HBVU6no.6; some stained cells can be seen but staining is less intense than with empty vector. (**f**) Liver section from a mouse that did not receive any pTHBV2. No staining was observed.

number of cells staining for HBcAg was substantially decreased (intensity of staining was also decreased).

HBVU6no.6 reduced the levels of the 2.4- and 2.1-kb RNAs and serum HBsAg even though it does not directly target these RNAs. Southern analysis showed a difference in the ratio of HBV-replicative molecular forms upon treatment with shRNAs. These data raise the possibility that shRNAs may have some sequence-independent antiviral effects at the dose used. Further studies will be required to determine the mechanism by which this occurs. As with other drugs, it is likely that there is a therapeutic window in which RNAi is effective but does not cause undesirable side effects. Further studies will also be required to determine if, at the optimal doses of RNAi, non-sequence-specific effects can be minimized or eliminated.

RNAi can theoretically be directed to cleave any target RNA, providing a single methodology for rational drug design for many different diseases. For this reason RNAi has generated substantial interest. It is clear from our study that inhibition of viral replication by RNAi in mammals is feasible. In the present study, six out of seven RNAi inhibitors tested showed some antiviral effect, and two out of seven were very potent inhibitors. However, a recent study found poliovirus escape mutants after extended treatment with siRNAs[11]. This suggests that multiple viral sequences must be targeted simultaneously so as to prevent the emergence of resistant strains.

METHODS

Plasmids. pTHBV2 (ref. 17) contains the HBV genome plus a redundancy for the sequences between nt 1067 and 1996 of the HBV genome. shRNAs were cloned downstream of the human U6 promoter as described[23]. Additional information on cloning and general structure of shRNA vectors can be found at http://katahdin.cshl.org:9331/RNAi/docs/Web_version_of_PCR_strategy1.pdf.

Target sequences are as follows:

HBVU6no.1, 5′-TCGTGGTGGACTTCTCTCAATTTTC-3′
HBVU6no.2, 5′-CTCAGTTTACTAGTGCCATTTGTTC-3′
HBVU6no.3, 5′-ATGATGTGGTATTGGGGGCCAAGTC-3′
HBVU6no.4, 5′-TGGCCAAAATTCGCAGTCCCCAACC-3′
HBVU6no.5, 5′-TCCCCGTCTGTGCCTTCTCATCTGC-3′
HBVU6no.6, 5′-CCTAGAAGAAGAACTCCCTCGCCTC-3′
HBVU6no.7, 5′-AGAAGATCTCAATCTCGGGAATCTC-3′

Negative control RNAi, 5′-TGGATATGCACGGTGTTGACTGATT-3′
pThAAT and pEmpty vector are described in refs. 18 and 14, respectively.

Statistical methods. A one-way ANOVA analysis with a post-hoc Fisher's test was conducted.

Cell culture and mouse transfections. Calcium phosphate transfections were carried out using standard methods. Hydrodynamic transfections of plasmids in PBS were carried out as described[20,21]. Mice that expressed very low levels of hAAT were considered poorly transfected and were excluded before analyses for HBV levels. Female BALB/c, C57BL/6J and NOD/LtSz-*Prkdc*^{scid}/J mice (18–22 g) were from Jackson Laboratory. Mice were treated according to NIH Guidelines for Animal Care and the Guidelines of Stanford University.

For descriptions of remaining procedures, see **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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